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Electronic Nose Technology for Detection of Invasive Pulmonary Aspergillosis in Prolonged Chemotherapy-Induced Neutropenia: a Proof-of-Principle Study

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Although the high mortality rate of pulmonary invasive aspergillosis (IA) in patients with prolonged chemotherapy-induced neutropenia (PCIN) can be reduced by timely diagnosis, a diagnostic test that reliably detects IA at an early stage is lacking. We hypothesized that an electronic nose (eNose) could fulfill this need. An eNose can discriminate various lung diseases through the analysis of exhaled volatile organic compounds (VOCs). An eNose is cheap and noninvasive and yields results within minutes. In a single-center prospective cohort study, we included patients who were treated with chemotherapy expected to result in PCIN. Based on standardized indications, a full diagnostic workup was performed to confirm invasive aspergillosis or to rule it out. Patients with no aspergillosis were considered controls, and patients with probable or proven aspergillosis were considered index cases. Exhaled breath was examined with a Cyranose 320 (Smith Detections, Pasadena, CA). The resulting data were analyzed using principal component reduction. The primary endpoint was cross-validated diagnostic accuracy, defined as the percentage of patients correctly classified using the leave-one-out method. Accuracy was validated by 100,000 random classifications. We included 46 subjects who underwent 16 diagnostic workups, resulting in 6 cases and 5 controls. The cross-validated accuracy of the eNose in diagnosing IA was 90.9% ($P = 0.022$; sensitivity, 100%; specificity, 83.3%). Receiver operating characteristic analysis showed an area under the curve of 0.93. These preliminary data indicate that PCIN patients with IA have a distinct exhaled VOC profile that can be detected with eNose technology. The diagnostic accuracy of the eNose for invasive aspergillosis warrants validation.

The diagnosis of pulmonary invasive aspergillosis (IA) poses a significant challenge in clinical practice due to the fact that symptoms and signs of it are neither sensitive nor specific (1, 2). This also holds for conventional chest X rays and cultures of sputum and/or bronchoalveolar lavage fluid specimens. Furthermore, computed tomography (CT) of the lungs is a sensitive but nonspecific test (3). The diagnosis is considered proven if a culture (of a specimen from a normally sterile site that is clinically or radiologically abnormal) yields *Aspergillus* spp. (3). Unfortunately, this requires invasive procedures, such as percutaneous or transbronchial lung biopsy, which are rarely possible for the majority of patients with IA, i.e., hematology patients experiencing prolonged chemotherapy-induced neutropenia. This is due to concurrent thrombocytopenia and the risk of pneumothorax, which is usually considered too high for these patients (4).

Over the past 10 years, a number of new tests have been introduced, most notably the Platelia assay, a double-sandwich enzyme-linked immunosorbent assay (ELISA) on galactomannan, a cell wall component of various molds, including *Aspergillus* spp. When performed with serum samples, the assay has sensitivity and specificity values of about 80%, and more importantly, a positive Platelia test can precede clinical manifestation with fever and other symptoms (5). It was shown recently that, when performed with bronchoalveolar lavage fluid specimens, the sensitivity and specificity of galactomannan are even higher (6). However, bronchoalveolar lavage is not without burden or even risks and often is not feasible. In addition, galactomannan is not detectable in serum until the accumulation of a considerable fungal burden. As the mortality rate of IA is high (>50%) and can be reduced by timely diagnosis, a diagnostic test that can reliably detect IA at an early stage remains one of the major goals in mycology and hematological supportive care (7–9).

Exhaled air is known to contain thousands of volatile organic compounds (VOCs) derived from various metabolic pathways (10). These VOCs can be used as biomarkers of lung disease, as has been demonstrated for bronchial carcinoma, infectious diseases, chronic obstructive pulmonary disease (COPD), and asthma (11–16). Recent evidence indicated that a specific VOC, 2-pentyl furan, might be a potential biomarker of IA (17, 18). However, the need for gas chromatography and mass spectrometry (GC-MS) in the assessment of individual volatile compounds precludes widespread on-site application in clinical practice.

An alternative way of assessing VOC mixtures is using electronic noses (eNoses). An electronic nose is an artificial olfactory system that discriminates complex odors using an array of sensors. When exposed to exhaled breath, the sensors react in a promiscuous way to the different fractions of VOCs (19–21). Each odor, which represents a unique mixture of VOCs, results in a pattern of sensor signals unique to that odor. This is called a "breathprint"

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when it concerns exhaled air. Using pattern recognition algorithms, complex mixtures of VOCs can thus be discriminated at high throughput without identifying the individual molecular components as such. eNoses are relatively cheap, mostly handheld, and easy to operate, and they yield results within minutes. From a patient's perspective, exhaled breath analysis is appealing because it is noninvasive, safe, rapid, simple to perform, and effort independent. Therefore, biomedical validation of eNoses is emerging (19, 22).

We hypothesized that exhaled breath analysis using an electronic nose (eNose) could be used to diagnose IA. To test that hypothesis, we performed a prospective proof-of-principle study.

MATERIALS AND METHODS

Subjects. Patients were included if they (i) were 18 years of age or older, (ii) had given written informed consent, and (iii) were treated for a hematological malignancy with chemotherapy expected to result in severe neutropenia ($<0.5 \times 10^9$ neutrophils/liter) for more than 7 days, e.g., hematopoietic stem cell transplantation or induction/consolidation treatment for acute myeloid leukemia. Patients were excluded if they were previously diagnosed with invasive mycosis or if they were unable to perform the breathing maneuver needed for eNose analysis of exhaled air. The Medical Ethics Committee of the Academic Medical Center approved the protocol of the study. The study was registered at ClinicalTrials.gov as study NCT01395446. All patients gave informed consent.

Design. This was a single-center prospective cohort study. Based on standardized indications, a full diagnostic workup was performed to confirm invasive aspergillosis or to rule it out. The results were classified according to the European Organization for Research and Treatment of Cancer (EORTC) criteria, revised in 2008 (3). In the event of no possible, probable, or proven aspergillosis and no seropositivity, the patient qualified as a neutropenic control. In the event of probable or proven aspergillosis, the patient qualified as a case. The breathprints of cases and controls were compared. Exhaled breath analysis was performed only once for each patient.

According to this design, patients with possible aspergillosis were excluded. We chose this design because of the proof-of-principle nature of our study. Patients with possible aspergillosis might truly have invasive aspergillosis but more often do not have invasive aspergillosis. Including patients with possible aspergillosis would make it harder to detect a breathprint associated with invasive aspergillosis.

Antifungal prophylaxis. All patients were managed identically according to a standardized protocol based on recent guidelines with respect to the prevention, diagnosis, and treatment of mycoses (23). Except for the analyses of exhaled air using the eNose, every prophylactic, diagnostic, and treatment-related procedure was performed according to standard care. Prophylactic antifungal treatment was started the same day as the chemotherapy. Each patient received 500 mg amphotericin B orally every 6 h until the peripheral neutrophil count exceeded 0.5×10^9 neutrophils/liter. Patients undergoing myeloablative allogeneic stem cell transplantation received 200 mg of fluconazole daily. If oral amphotericin B was not tolerated, no substitute was started. In principle, no antimycotic with activity against *Aspergillus* spp., such as voriconazole or posaconazole, was administered prophylactically. If the treating physician judged that the administration of prophylactic antimold treatment was necessary, then the patient was excluded from the study, as eNose results can be influenced by antimold therapy.

Diagnostic strategy. From the start of chemotherapy, cultures of the throat, nose, rectum, and, if possible, sputum specimens were performed weekly. From the start of neutropenia ($<0.5 \times 10^9$ neutrophils/liter), a serum galactomannan assay was performed twice weekly. Both procedures were continued until the peripheral neutrophil count exceeded 0.5×10^9 neutrophils/liter. A complete diagnostic workup was performed in the case of a number of standardized indications for a diagnostic workup, based on international guidelines: (i) a rise of serum galactomannan levels above an optical density index of 0.5, (ii) $\geq$5 days of fever that was unresponsive to broad empirical antibiotic treatment and without an alternative explanation, (iii) a new infiltrate developing under broad antibiotic coverage or high-dose steroid treatment, (iv) an abnormality on a chest X ray consistent with invasive pulmonary mycosis, (v) hyphae or molds found in a respiratory tract specimen, or (vi) symptoms and/or signs considered by the treating physician to be possibly due to invasive mycosis (23).

Diagnostic workup. The workup consisted of (i) analysis of sputum specimens (using direct microscopy and culture), (ii) high-resolution CT of the thorax, and (iii) in the case of abnormalities on the high-resolution CT scan consistent with invasive pulmonary mycosis, bronchoscopy and bronchoalveolar lavage (BAL). BAL fluid specimens were examined using direct microscopy; PCR for the *Mycobacterium tuberculosis* complex, mycobacterial culture, PCRs for respiratory viruses (human bocavirus, parainfluenza 1 to 4, parechovirus, coronavirus, rhinovirus, respiratory syncytial virus, human metapneumovirus, enterovirus, influenza A and B viruses, adenovirus, herpes simplex virus, Epstein-Barr virus, and cytomegalovirus), routine cultures, and measurement of galactomannan were performed as well. If bronchoalveolar lavage was not performed, then a throat gargle specimen was examined using PCRs for the above-mentioned respiratory viruses. Sinonasal, ophthalmological, and neurological symptoms and signs were actively sought. On indication, CT of the liver and spleen, CT or magnetic resonance imaging (MRI) of the brain and sinuses, or consultation with a neurologist, otolaryngologist, or ophthalmologist was performed.

Exhaled breath analysis. Every diagnostic workup was followed by exhaled breath analysis, as described previously (11, 24). Patients were asked to breathe through a mouthpiece for 5 min with their nose clipped. Through a three-way nonrebreathing valve, this mouthpiece was connected to an inspiratory VOC filter (A2; North Safety, Middelburg, The Netherlands) as well as an expiratory silica reservoir. Then, a deep inspiratory capacity maneuver was followed by exhalation of a vital capacity volume. The exhaled breath was collected in a 10-liter Tedlar bag connected to the silica reservoir. Within 30 min, the Tedlar bag was sampled using the electronic nose, a Cyranose 320 (Smith Detections, Pasadena, CA). This is a handheld chemical vapor analyzer based on a nanocomposite sensor array with 32 polymer sensors (19). The changes in electrical resistance in each of the 32 sensors were stored as raw data for further analysis. Every sampling procedure was repeated, after which the first measurement was disregarded as described previously, because of deviant raw data in the first run (11).

Analysis. As our primary analysis, we compared the breathprints of cases and controls. We performed offline analysis of the raw data using R (version 2.11.1). First, data reduction by principal component analysis (PCA) was performed to reduce the original data from the 32 sensors to a nonpredefined number of principal components, capturing at least 99.9% of the variance within the data set. Second, *t* tests (equal variance assumed, depending on the outcome of an *F* test) were used to assess which PCA factors discriminated between the two groups; two-sided *P* values of 0.10 were considered significant. Then, based on the differentiating PCA factors, a categorical division was made using linear canonical discriminant analysis, assuming equal chances of being a member of one of the two groups. The discriminant function was chosen to distinguish best between categories. Finally, the accuracy of this model was established. This was defined as the percentage of correctly classified patients, with cases and control subjects combined. Cross-validation using the leave-one-out method was used to calculate the cross-validated accuracy. The 95% confidence intervals (CIs) were calculated using the exact binomial test. To calculate our *P* value, we generated 100,000 random classifications of our subjects ("whether or not a case or control") and determined the chance that a random classification would have led to a cross-validated accuracy identical to our primary outcome or better, constructing a new pattern recognition algorithm for each of the random classifications using the

TABLE 1 Subject characteristics

Aspergillosis status and subject no.	Age (yr)	Sex	Diagnosis[a]	Therapy[b]	HR CT of the lungs[c]	Galactomannan level (ng/ml) in[d]: Serum	Galactomannan level (ng/ml) in[d]: BAL fluid	Cultures positive for *Aspergillus* spp.	EORTC classification	Remarks
Probable/proven aspergillosis										
1	62	F	AML	Induction	P	0.1	2.3	No	Probable	Concurrent influenza (H1N1)
3	35	F	AML	Induction	P	0.2	7.4	No	Probable	
4	47	M	AML	Induction	P	1.9	7.6	No	Probable	
8	70	M	ALL	Induction	P	0.3	8.1	No	Probable	
9	56	M	Waldenström	R-VIM	P	1.2	n.p.	No	Probable	
Mean[e]	54.0					0.7	6.4			
No aspergillosis										
2	53	F	AML	Induction	N	0.1	n.p.	No	No	
5	51	F	AML	Induction	N	0.2	n.p.	No	No	
6	65	F	AML	Induction	P	0.1	0.3	No	No	Bacterial pneumonia
7	46	F	AML	Induction	N	0.1	n.p.	No	No	Left-sided pleural fluid
10	63	M	AML	Induction	N	0.1	n.p.	No	No	Bacterial pneumonia
11	74	M	AML	Induction	N	0.1	n.p.	No	No	Bilateral pleural fluid
Mean[f]	58.7					0.1	0.3			

[a] AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.
[b] Induction, induction chemotherapy; R-VIM, rituximab-etoposide-iphosphamide-methotrexate.
[c] HR, high-resolution; P, positive as a clinical EORTC criterion; N, negative as a clinical EORTC criterion.
[d] n.p., not performed.
[e] The group was 40% female.
[f] The group was 67% female.

statistical method of the primary analysis (25). Finally, receiver operating characteristic (ROC) analysis was performed.

RESULTS

During the study period, there were 53 eligible patients. As 5 refused to provide informed consent and 2 had been diagnosed previously with invasive mycosis, 46 patients were included. For 16 of these subjects, one or more triggers for a diagnostic workup occurred. This resulted in 6 controls and 5 cases (Table 1). Principal component analysis of the raw data resulted in 8 principal components (PCs) that described 99.9% of the variance. Of these, 1 discriminated between cases and controls. Subsequent canonical discriminant analysis showed a cross-validated accuracy value of 90.9% (95% CI, 59% to 100%). The sensitivity and specificity were 100% (95% CI, 48% to 100%) and 83.3% (95% CI, 36% to 100%), respectively. Figure 1 shows the individual discriminant scores. ROC analysis of the discriminant scores revealed an area under the curve of 0.933. In our simulation, 2.2% of the 100,000 random classifications resulted in cross-validated accuracy of ≥90.9%. For all patients for whom both values were determined, we calculated the correlation between the discriminant scores and BAL fluid galactomannan levels, currently the most accurate single test to diagnose invasive pulmonary aspergillosis. Although these values were correlated, this correlation was not statistically significant (unstandardized regression coefficient, −0.23 [95% CI, −0.54 to 0.08]; $R^2 = 0.36$) (Fig. 2).

DISCUSSION

Our study shows that patients with invasive aspergillosis have an exhaled VOC profile distinct from the findings for controls, which can be established by eNose technology. The accuracy is high, and as shown by the random classifications, this is not a coincident finding. This implies that in the future, exhaled breath analysis might become a noninvasive addition to the diagnostic arsenal for invasive aspergillosis that is cheap, fast, and simple to perform.

We hope that eNose technology will enable us to detect invasive aspergillosis at an earlier point in time than do currently available diagnostic tools. At the time of the exhaled breath analysis for subject 4, he was thought to have no possible, probable, or proven aspergillosis, based on the diagnostic workup according to protocol. Two weeks later, however, probable aspergillosis was diagnosed. In retrospect, very small pulmonary lesions were visible 2 weeks earlier at the locations where aspergilloma later developed. Therefore, the subject was classified as being a case in our study. Out of interest, we performed a second exhaled breath analysis 2 weeks later, when we diagnosed probable aspergillosis. This measurement was not used to derive the pattern recognition algorithm for our primary analysis, of course. We compared the two exhaled breath analyses. Although the first signal (discriminant score, −0.49) was less pronounced than the second (discriminant score, −1.37), it did already indicate IA.

To our knowledge, this is the first study examining the accuracy of exhaled air analysis in the early diagnosis of invasive aspergillosis. It is, however, in line with previous *in vitro* research, which already showed that an eNose can reliably differentiate *in vitro* the most frequently encountered pathogens in pneumonia. Moens et al. demonstrated that an eNose can differentiate, with a diagnostic accuracy of 100%, the headspaces of various microorganisms after 17 h of culturing (26). Those authors examined Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Proteus vulgaris*), Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*), a yeast species (*Candida albi-*

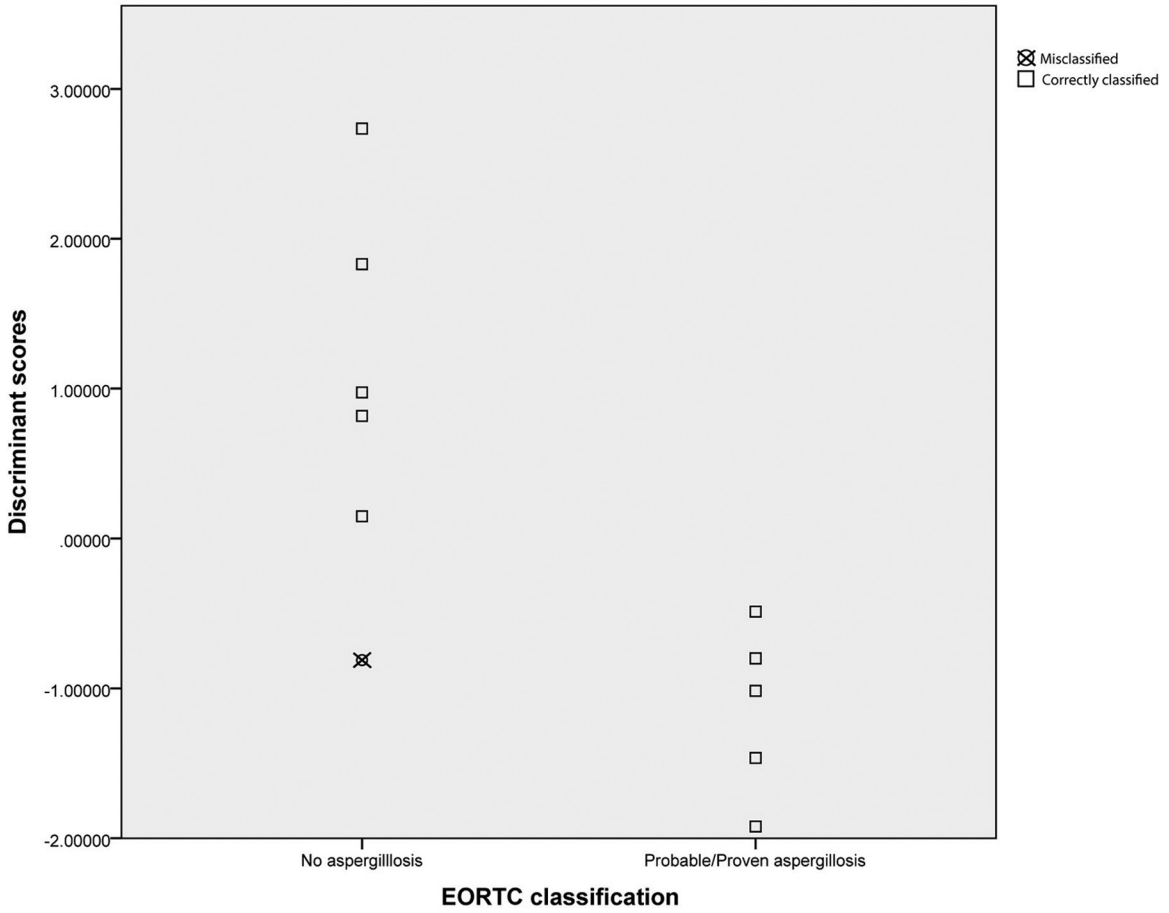

FIG 1 Individual discriminant scores derived from exhaled breath profiles of patients with and without invasive pulmonary aspergillosis.

cans), and a mold species (*Aspergillus fumigatus*) (26). Other groups have confirmed that an eNose is able to differentiate the headspaces of various microorganisms (27, 28). These results were already extended to an *in vivo* situation, i.e., ventilator-associated pneumonia. Hockstein et al. calculated pneumonia scores for 44 ventilated patients based on a number of clinical criteria (29, 30). An eNose reliably differentiated between the 7 patients with high pneumonia scores and the 29 patients with low pneumonia scores. Our data thus support and extend the accumulating evidence that eNose technology can be used to diagnose pulmonary infections.

Our study has a number of strong points. We studied a prospective cohort in which the patients were followed according to a state-of-the-art diagnostic protocol, defining the timing of our exhaled breath analyses and characterizing our population well with respect to whether aspergillosis occurred. This protocol also yielded a well-characterized control group.

In contrast, our study is subject to two major limitations. First, the sample size was small due to the low incidence of IA. This precluded external validation of our results. However, our 100,000 random classifications indicated that the chance of false-positive discovery was only 2.2%. Eventually, according to guidelines on stepwise assessment of the diagnostic accuracy of novel tests, confirmation of our results with a separate group of subjects not involved in generating the pattern recognition algorithm will be required to definitively establish the ability of an eNose to detect IA (31). Such external validation has already been provided for eNose differentiation between COPD and asthma (32).

Second, eNose technology, albeit applicable for medical applications, does not allow identification of the individual VOCs that drive the signal. It is unknown which VOCs enable the detection of IA by eNose technology. These might be VOCs produced by *A. fumigatus* itself. In the literature, a number of potential candidates have been suggested. One such compound is 2-pentyl furan, which was reported by a research group from New Zealand to be *A. fumigatus* specific and exhaled by subjects with colonization as well as invasive disease caused by *A. fumigatus* (17, 18). However, differences in the composite molecular signatures captured by breathprints may arise from sources other than *A. fumigatus*, such as host responses. The presence of *Aspergillus* spp. in the airways triggers an immune response. In a number of patients, this even leads to the clinical entity called allergic bronchopulmonary aspergillosis (33). Notably, inflammatory airway diseases, such as asthma and COPD, can be discriminated at a high level of accuracy through eNose technology, in which the signals detected by an eNose as well as GC-MS are significantly associated with cellular and molecular markers of airway inflammation (34, 35). Such inflammatory host responses might have played a major role in our study, augmenting the difference in exhaled VOC profiles and aiding early detection. Invasive aspergillosis induces a major immune response, despite neutropenia (36).

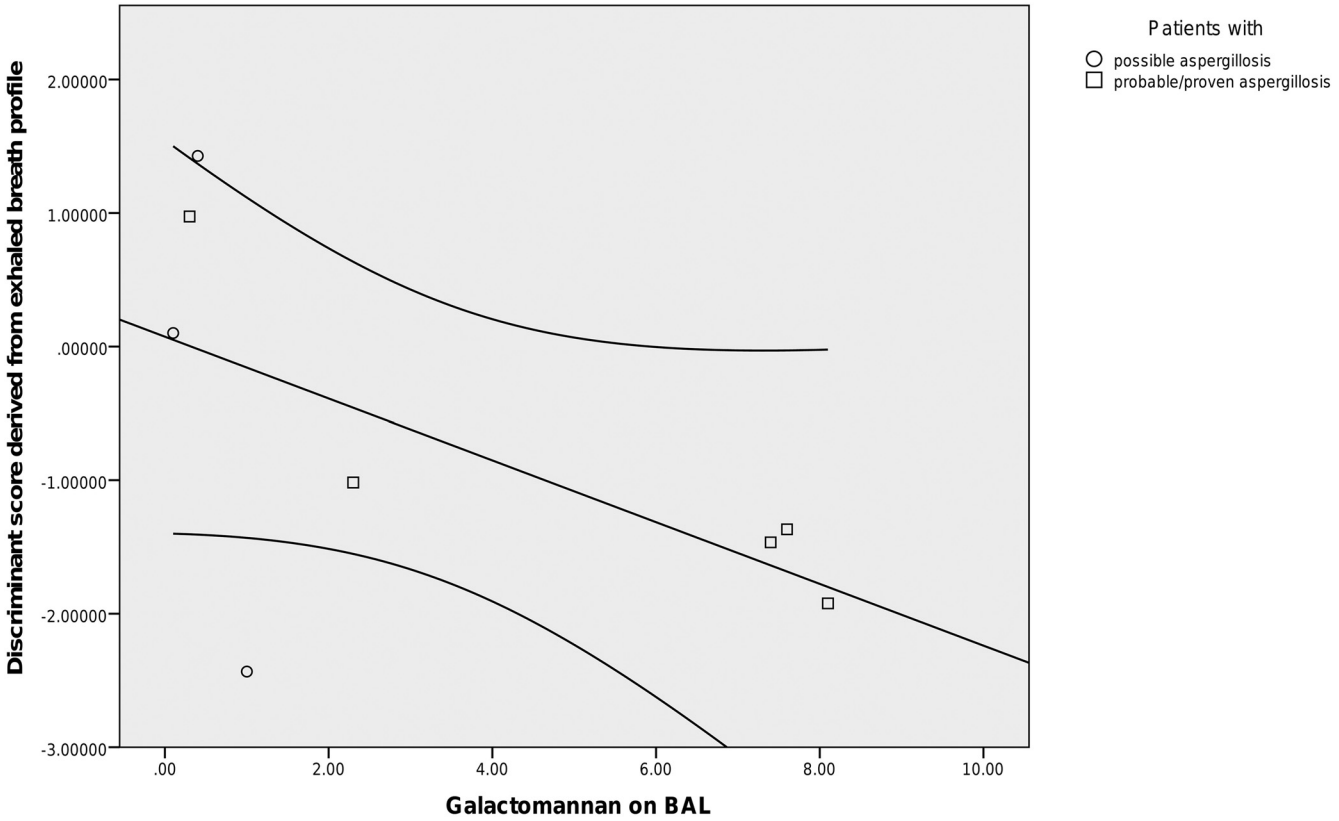

FIG 2 Correlation of galactomannan levels in BAL fluid and discriminant scores based on exhaled breath profiles for subjects for whom bronchoscopy was performed. The fit line based on linear regression and the 95% mean prediction intervals are shown.

The implications of our results are potentially broad. Exhaled breath analysis could increase the accuracy of the diagnostic workup of patients suspected of having invasive aspergillosis. It might also decrease the mortality rate of invasive aspergillosis, for example, through a reduction of the diagnostic delay with monitoring of patients with prolonged chemotherapy-induced neutropenia twice per week. Lastly, if it were to improve diagnostic accuracy enough, it could obviate bronchoscopy, thereby making the workup less invasive. Furthermore, if further translational research identifies the molecules involved in the generation of the specific breathprint, then eNoses could be "tailor made" to detect those VOCs to improve diagnostic accuracy even further (16).

In conclusion, this study shows the potential of eNose technology in the detection of IA in patients experiencing prolonged chemotherapy-induced neutropenia through the analysis of exhaled breath. This warrants the next step in the testing of diagnostic accuracy: performing a large-scale validation study in order to determine how much diagnostic delay can be prevented by adding twice-weekly exhaled breath analyses using an eNose to a state-of-the-art diagnostic strategy for invasive aspergillosis (31).

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